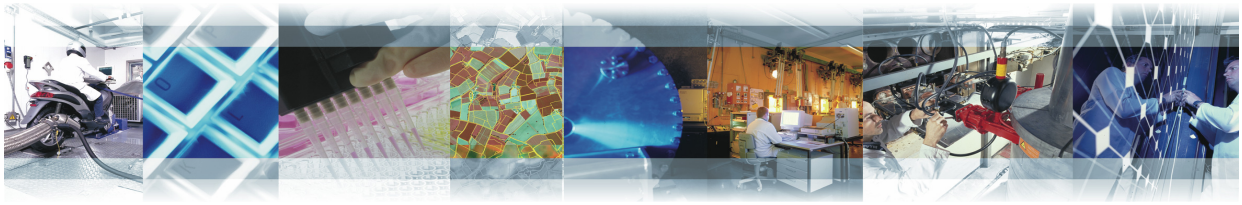

Foodstuffs - Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection

Validated method

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Contents

Page

Foodstuffs - Simultaneous determination of nine intense sweeteners by HPLC-ELSD

1 Scope

This standard specifies a high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD) for the simultaneous determination of nine intense sweeteners, i.e., acesulfame-K (ACS-K), alitame (ALI), aspartame (ASP), cyclamic acid (CYC), dulcin (DUL), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin (SAC) and sucralose (SCL), in beverages and canned or bottled fruits.

2 Principle

The procedure involves extraction of the nine sweeteners with a buffer solution, sample clean-up using solid-phase extraction cartridges followed by HPLC-ELSD analysis.

3 Reagents, solutions and standards

Use only reagents of recognized analytical grade, unless otherwise stated.

3.1 **Acesulfame-K**, adequate purity (e.g. Fluka, DE).

3.2 **Alitame**, adequate purity (e.g. could be obtained from producers).

3.3 **Aspartame**, adequate purity (e.g. Supelco, DE or LGC Promochem, UK).

3.4 **Dulcin**, adequate purity (e.g. could be obtained from producers).

3.5 **Neotame**, adequate purity (e.g. LGC Promochem, UK).

3.6 **Neohesperidine dihydrochalcone**, adequate purity (e.g. Sigma-Aldrich, DE).

3.7 **Saccharin, sodium salt dehydrate**, adequate purity (e.g. Sigma-Aldrich, DE).

3.8 **Sodium cyclamate**, adequate purity (e.g. Merck Schuchardt OHG, DE).

3.9 **Sucralose**, adequate purity (e.g. LGC Promochem, UK).

3.10 **Formic acid** (puriss. p.a. ~ 98 %).

3.11 **Water** (HPLC grade).

3.12 **Triethylamine** (puriss. p.a. > 99.5 %).

3.13 Methanol (HPLC grade).

3.14 Acetone (HPLC grade).

3.15 Buffer solution (pH = 4.5).

Dissolve 4 mL of formic acid (3.10) in 5 L of water (3.11). Adjust to pH 4.5 with ca. 12.5 mL triethylamine (3.12).

3.16 HPLC mobile phase A, methanol – buffer solution – acetone 69:24:7 (v/v/v)

Mix 690 mL of methanol (3.13) with 240 mL of buffer solution (3.15) and with 70 mL of acetone (3.14). Degas by sonication for 10 minutes.

3.17 HPLC Mobile phase B, methanol - buffer solution – acetone 11:82:7 (v/v/v)

Mix 110 mL of methanol (3.13) with 820 mL of buffer solution (3.15) and with 70 mL of acetone (3.14). Degas by sonication for 10 minutes.

3.18 Mixed stock standard solution, ACS-K, ALI, ASP, CYC-Na, DUL, NEO, NHDC, SAC-Na and SCL; $c_{(\text{sweetener i})}$ ~ 30 - 250 µg/mL

Prepare a mixed stock standard solution of all nine sweeteners by weighing the given masses of the individual sweetener standards (Table 1) first into a 100 mL beaker and dissolving them in approximately 50 mL of methanol:water (1:1) until complete dissolution. Then transfer the obtained solution quantitatively into a 500 mL volumetric flask and make up to the mark with the buffer solution (3.15). Mix thoroughly by sonication until complete dissolution.

Note: In case of cyclamic acid and saccharin, their sodium salts are used, since they are either not available in free form or poorly soluble.

Note: The final concentrations of the individual sweeteners (µg/mL) in the mixed stock standard solution have to be calculated by using the actually weighed masses.

Table 1. Masses of individual standards for preparation of mixed stock standard solution

Standard	Mass [mg] weighed into 500 mL volumetric flask [3]	Final concentration of sweetener i in mixed stock standard [µg/mL]
Acesulfame-K (ACS-K)	45	90
Alitame (ALI)	25	50
Aspartame (ASP)	125	250
Sodium cyclamate (CYC-Na) **Cyclamic acid (CYC) (free acid)**	140 [1] –	– 249.42
Dulcin (DUL)	25	50
Neotame (NEO)	25	50
Neohesperidine dihydrochalcone (NHDC)	15	30
Saccharin, sodium salt dihydrate (SAC-Na·2H$_2$O) **Saccharin (SAC) (free imide)**	35 [2] –	– 53.17
Sucralose (SCL)	50	100

[1] equivalent to 124.71 mg free cyclamic acid;
conversion factor to calculate mass of free cyclamic acid = 0.8908;
$m_{CYC} = 0.8908 \times m_{CYC\text{-}Na}$
[2] equivalent to 26.58 mg free saccharin;
conversion factor to calculate mass of free saccharin = 0.7595;
$m_{SAC} = 0.7595 \times m_{SAC\text{-}Na\cdot 2H2O}$
[3] first weigh into 100 mL volumetric flask, dissolve in approximately 50 mL of a methanol:water (1:1) mixture and then transfer quantitatively into 500 mL volumetric flask

3.19 Calibration standard solutions

From the mixed stock standard solution (3.18) prepare a series of calibration standard solutions containing the sweeteners at levels fitting appropriate limits, e.g., the highest concentration of the calibration shall be at least equivalent to 125 % of the given limits, such as those in Commission Directives 94/35/EC [1] as amended by Directives 96/83/EC [2] and 2003/115/EC [3] (see Table 2), whilst taking the dilution steps within the procedure into account (see Table 3). For unauthorised sweeteners (ALI, DUL and NEO) fictitious MUDs were assumed at ca. 200 mg/L or mg/kg.

Table 2: Present EU limits for the nine sweeteners in beverages and canned fruits

Sweetener	MUD [1] for beverages [mg/L]	MUD [1] for canned fruits [mg/kg]
ACS-K	350	350
ALI [2]	-	-
ASP	600	1000
CYC	250	1000
DUL [2]	-	-
NEO [2]	-	-
NHDC	30	50
SAC	80	200
SCL	300	400

[1] MUD = maximum usable dosage according to present EU limits [1-3]
[2] unauthorised sweeteners according to present EU limits [1-3]

Note: The present procedure is simplified by preparing one calibration series for both food matrices. The described calibration series is fitted to canned fruits as the MUDs for canned fruits are in some cases higher than the MUDs for beverags. In case only the latter matrix is analysed the calibration series can be fitted to the MUDs of beverages.

Pipette the following volumes (see Table 3) from the mixed stock standard solution (3.18) into appropriate volumetric flasks (10 - 50 mL) and make up to the mark with buffer solution (3.15) and shake thoroughly.

Table 3. Preparation of series of calibration standard solutions

Calibration solution	Volume of volumetric flask [mL]	Volume taken from mixed stock standard solution (3.18) [mL]	Volume taken from buffer solution (3.15) [mL]
1 [(1)]	10	10	0
2	10	8	2
3	10	6	4
4	10	4	6
5	10	2	8
6	25	3	22
7	50	3	47
8	50	1.5	48.5

[(1)] undiluted mixed stock standard solution (3.18)

Table 4 details the concentration of sweetener i in each calibration standard following preparation described in Table 3.

Table 4. Concentration of sweetener i in the individual calibration standard solutions

	Calibration solution							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sweetener	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**
ACS-K	90.0	72.0	54.0	36.0	18.0	10.8	5.4	2.7 [(1)]
ALI	50.0	40.0	30.0	20.0	10.0	6.0	3.0 [(1)]	1.5 [(1)]
ASP	250.0	200.0	150.0	100.0	50.0	30.0	15.0	7.5
CYC	249.4	199.5	149.7	99.8	49.9	29.9	15.0	7.5
DUL	50.0	40.0	30.0	20.0	10.0	6.0 [(1)]	3.0 [(1)]	1.5 [(1)]
NEO	50.0	40.0	30.0	20.0	10.0	6.0	3.0 [(1)]	1.5 [(1)]
NHDC	30.0	24.0	18.0	12.0	6.0	3.6 [(1)]	1.8 [(1)]	0.9 [(1)]
SAC	53.2	42.5	31.9	21.3	10.6	6.4	3.2 [(1)]	1.6 [(1)]
SCL	100.0	80.0	60.0	40.0	20.0	12.0	6.0	3.0 [(1)]

[(1)] the concentration level might be below the limit of quantification (LOQ). If yes, the result obtained by HPLC analysis is not included in the construction of the calibration graph, e.g., in case of ACS-K a seven point calibration is performed, ignoring the result obtained for calibration solution 8. The results can differ from laboratory to laboratory.

4 Apparatus and equipment

Usual laboratory equipment and, in particular, the following:

4.1 Common laboratory glassware, such as graduated cylinders, volumetric pipettes, glass beakers etc.

4.2 Analytical balance, capable of weighing to 0.01 mg.

4.3 Laboratory balance, capable of weighing to 0.01 g.

4.4. Positive displacement pipette, or equivalent**,** capable of delivering 1-10 mL (variable volume).

4.5 Volumetric flasks, of 10 mL, 25 mL, 50 mL, 100 mL and 500 mL capacity.

4.6 Centrifuge tubes, polypropylene, 50 mL capacity.

4.7 Graduated test tubes, 5 mL capacity.

4.8 Food blender, suitable for homogenisation of food samples (e.g. Grindomix GM200, Retsch).

4.9 Ultrasonic bath.

4.10 Centrifuge, capable of maintaining 4000 rpm.

4.11 SPE Vacuum system, or equivalent.

4.12 Equipment for solvent evaporation.

4.13 pH meter.

4.14 C18 SPE cartridges, such as Chromabond® C18ec, 6 mL/1000 mg (Macherey-Nagel, or equivalent).

4.15 Fully end-capped reversed phase HPLC analytical columns of 250 mm x 3 mm dimensions, particle size 5 µm, allowing sufficient separation of all nine sweeteners. Suitable columns are

- Zorbax Extend-C18 (Agilent)
- Purospher® Star RP-18 (Merck)
- Nucleodur® C18 Pyramid (Macherey-Nagel)
- Nucleodur® C8 Gravity (Macherey-Nagel).

4.16 HPLC system, equipped with a binary pump capable of maintaining a flow rate of 0.5 mL/min, preferably an automatic injection system, and an evaporative light scattering detector (e.g. Alltech ELSD 2000ES or equivalent).

4.17 Data acquisition and analysis software.

5 Sampling

Sampling is not part of this method.

6 Procedure

6.1 Preparation of test sample

Comminute the entire test sample to give a homogenous suspension (4.8). Liquid samples may be subjected directly to the extraction procedure.

6.2 Extraction and clean-up

6.2.1 Weigh ca. 5 g (M1, recorded to 2 decimal places) of the homogenised test sample (6.1) into a volumetric flask of 50 mL (V1). Make up to the mark with buffer solution (3.15), mix thoroughly by hand to obtain a homogeneous suspension and sonicate (4.9) for 15 min.

6.2.2 Transfer the obtained suspension to a 50 mL centrifuge tube. Centrifuge at 4000 rpm for 10 min.

Note: In case the test sample gives a clear solution (e.g. some beverages), this step can be ignored.

6.2.3 Condition the SPE cartridges (4.14) by applying 3 mL methanol (3.13) and let it pass through using a slight vacuum resulting in a flow rate of 1-2 mL/min. Make sure that a small portion of methanol remains above the sorbent bed (1 mm).

6.2.4 Equilibrate the SPE cartridges by applying 2 mL of buffer solution (3.15) and let it pass through using a slight vacuum resulting in a flow rate of 1-2 mL/min. Make sure that a small portion of buffer solution remains above the sorbent bed (1 mm). Repeat the procedure two times.

6.2.5 Load the SPE cartridges with 5 mL of sample extract (V2 first loading), i.e., the supernatant from (6.2.2), and let it pass through using a slight vacuum resulting in a flow rate of 1-2 mL/min. Make sure that a small portion remains above the sorbent bed (1 mm). Repeat the procedure once more (V2 in total 10 mL).

6.2.6 Wash the SPE cartridges with 3 mL of buffer solution (3.15) and let it pass through using a slight vacuum resulting in a flow rate of 1-2 mL/min. Make sure that a small portion of buffer solution remains above the sorbent bed (1 mm).

6.2.7 Elute the sweeteners from the SPE cartridges by applying 2 mL of methanol (3.13) and collecting the eluate in a 5 mL test tube. Use a slight vacuum to obtain a flow rate of 1 mL/min. Make sure that a small portion of methanol remains above the sorbent bed (1 mm). Wait 10 min before applying a second portion of 2 mL of methanol (3.13) and elute it subsequently to the same 5 mL test tube using the same vacuum conditions but this time letting the SPE cartridges run dry.

Note: Avoid in all steps (6.2.1 to 6.2.7) that the sorbent bed runs dry with the only exception of the last step, i.e., second elution of analytes (6.2.7).

6.2.8 Evaporate the solvent from the methanolic SPE extract to 3 mL under a stream of nitrogen at ambient temperature.

Note: Temperatures above 40 °C have to be avoided, since aspartame can degrade.

6.2.9 Fill the graduated test tube containing the SPE extract (6.2.8) up to the 5 mL mark with buffer solution (3.15) (V3). Mix thoroughly and transfer the content into a suitable HPLC vial and analyse by HPLC.

6.3 HPLC conditions

Establish suitable HPLC conditions to meet the predefined performance criteria (8.2). The separation and quantification have proven to be satisfactory if the following experimental conditions are followed:

- Column: see 4.15
- Column temperature: ambient temperature
- Injection volume: 10 µL
- Mobile phase: see 3.16 and 3.17
- Mobile phase flow rate: 0.5 mL/min
- Separation mode: gradient
- Gradient program:

Time [min]	0	4	11	23	24	26	36
Mobile phase A [%]	0	0	53	100	100	0	0
Mobile phase B [%]	100	100	47	0	0	100	100

- Detector: evaporative light scattering detector (ELSD)
- ELSD drift tube temperature: 85 °C
- ELSD nitrogen flow: 2.5 L/min

- ELSD gain: 1
- ELSD impactor: Off

Note: The given detector parameters are applicable to the Alltech ELSD 2000ES system. Alternative ELSD systems and experimental conditions, used in an interlaboratory study, are listed in Annex A, Table A 1. HPLC and ELSD operating conditions may be changed to obtain optimum separation.

6.4 HPLC sequence

Single, double or triple injection per sample should be performed according to the needs. The sequence has to include:

- 8 calibration standard solutions differing in concentration level (3.19)
- test sample(s)
- after every 20th test sample an extra series of calibration standard solutions shall be analysed (3.19).

Note: For screening purpose, the sequence of injection can be different from the sequence mentioned above.

6.5 Construction of calibration graph

Analyse the eight calibration standard solutions (3.19, Table 3) using HPLC conditions identical to those used for the test samples (6.3), i.e., inject 10 µL of each solution into the HPLC system. Construct a calibration chart for each sweetener i from the results of the analysis of the standard solutions. Plot the obtained peak area as log_{10}*(Peak area i)* (y-axis) against the log_{10}*(Concentration i)* (x-axis) (Figure 1). Fit a straight line (y = a + bx) to the results, where *b* is the value of the slope of the linear function and *a* is the value where the calibration function intercepts the y-axis. If the results of the analyses of the standard solutions are linear, the calibration line may be used to calculate the concentration of sweetener i in the sample extract.

Note: The calibration graphs of the nine sweeteners can differ in the number of calibration points used (3.19, see Table 4), e.g., ACS-K (seven point calibration), ALI (six point calibration), ASP (eight point calibration), CYC (eight point calibration), DUL (five point calibration), NEO (six point calibration), NHDC (five point calibration), SAC (six point calibration), SCL (seven point calibration). Examples of the individual calibration graphs of all nine sweeteners are given in Figures B 1-9 (Annex B).

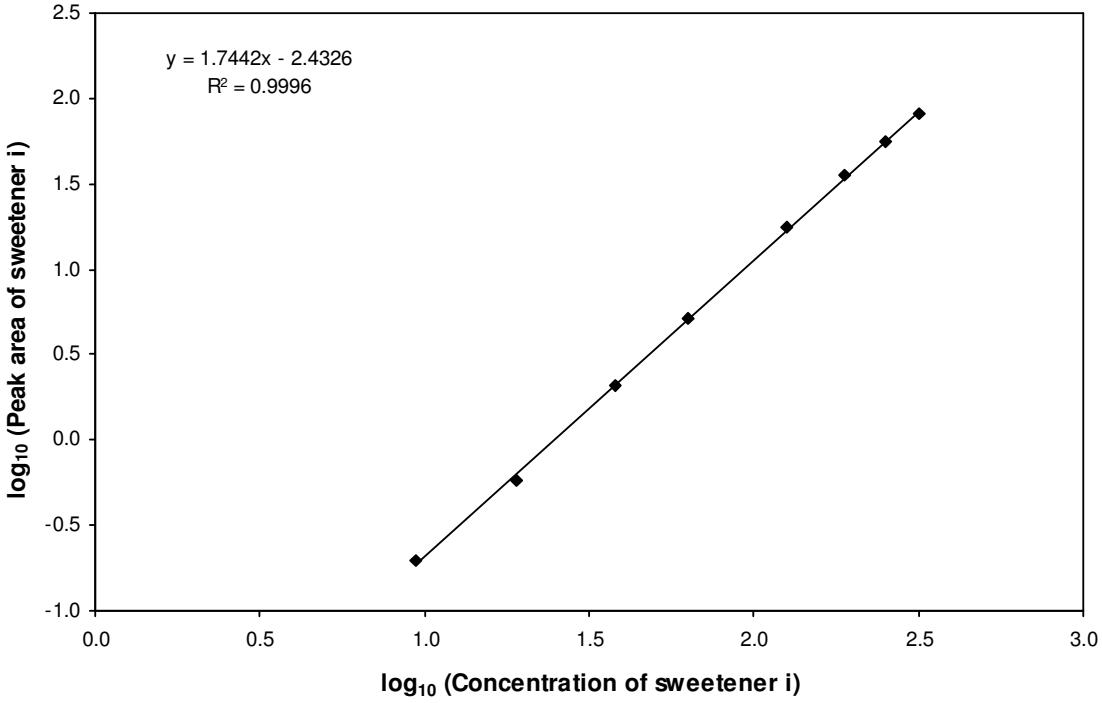

Figure 1. Example of calibration graph for sweetener i, for which *a* results in -2.4326 and *b* in 1.7442

6.6 HPLC analysis of test sample

Analyse 10 µL of the sample extract solution (6.2.9).

6.7 Interpretation of chromatographic data

6.7.1 Identify the individual sweeteners in the test samples by comparison of the retention time of sweeteners observed during the analysis of standard solutions analysed in the same batch as samples with the retention time of compounds eluted during the analysis of the test samples. The elution order of the individual sweeteners together with the retention times are given in an example chromatogram in Figure C 1 (Annex C).

6.7.2 Measure the peak area response (Ri) observed for sweetener i in each solution. In case the peak area of sweetener i in the chromatogram of the test sample solution exceeds the area of the respective sweetener peak in the chromatogram obtained for the calibration standard solution with the highest concentration, the test sample solution is diluted with buffer solution (3.15) and the diluted extract re-analysed.

7 Calculation of results

Quantitative determination of sweetener i is carried out by integration of the peak area i (R_i) (6.7.2) obtained from the analysis of the injected SPE extract (6.6). Use the resulting calibration function, i.e., y = bx + a (6.5) to calculate the concentration of sweetener i (C_{1i}) in the measured sample extract solution using equation 1 and 2.

Equation 1. $$\log_{10} C_{1i} = \frac{(\log_{10} R_i) - a_i}{b_i}$$

Equation 2. $$C_{1i}\,[\mu g/mL] = 10^{(\log_{10} C_{1i})}$$

where

R_i	is the peak area response (6.7.2) for sweetener i
a_i	is the intercept of the calibration line (6.5) for sweetener i
b_i	is the slope of the calibration line (6.5) for sweetener i
C_{1i}	is the concentration of sweetener i in the SPE extract [µg/mL]

Calculate the concentration/mass fraction of sweetener i in the test sample according to equation 3.

Equation 3. $$C_{2i}\left[\frac{\mu g}{g}\right] = \frac{C_{1i} \text{ x } V_1 \text{ x } V_3}{M_1 \text{ x } V_2}\left[\frac{\mu g \text{ x mL x mL}}{mL \text{ x g x mL}}\right]$$

where

C_{1i}	is the concentration of sweetener i in the SPE extract [µg/mL] (as determined in Equation 2)
C_{2i}	is the mass fraction of sweetener i in the sample [µg/g]
M_1	is the mass of the sample taken for extraction [g], i.e., 5 g (6.2.1)
V_1	is the total volume of the sample solution [mL], i.e., 50 mL (6.2.1)
V_2	is the volume of the sample solution loaded onto the SPE cartridge [mL], i.e., 10 mL (6.2.5)
V_3	is the final volume of the SPE extract [mL], i.e., 5 mL (6.2.9)

8 Procedural requirements

8.1 General

The details of the chromatographic procedure depend, among other factors, on equipment, type, age, and supplier of the column, sample size and detector. Different columns may be used, and injection volumes may be varied, if the requirements of the system suitability tests are met.

8.2 System suitability test – Resolution of separation system

The HPLC-ELSD system shall be capable of separating all nine sweeteners from each other with at least baseline separation. This requirement can be proven by using calibration solution 1 (3.19) as shown in Figure B 1 (Annex B).

Moreover, the system shall be capable of separating all nine sweeteners from other components of the matrix. Many matrix components, such as sodium benzoate, sorbic acid, citric acid, phosphoric acid, malic acid, ascorbic acid, glutamic acid, sucrose, glucose, fructose, lactose, caffeine, taurine, D-glucurono-γ-lactone and sorbitol, etc. are removed throughout the SPE clean-up. A commonly encountered critical pair is alitame (unauthorised sweetener) and quinine, which is not removed by the SPE clean-up [4].

NOTE: In case of failure, the chromatographic conditions (e.g. sample volume injected, mobile phase rate, gradient program, etc.) or the ELSD conditions (e.g. drift tube temperature, nitrogen/air flow) must be optimized.

9 Precision

9.1 General

Details of the of the methods used by the individual laboratories in the interlaboratory test are listed in Table A 1 in Annex A, and the composition of the individual test materials used are listed in Tables A 2-3 in Annex A. Precision data of the method are summarized in Tables A 4-12 in Annex A. The values derived from this interlaboratory study test may not be applicable to concentration ranges and matrices other than those given.

9.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the

repeatability limit r as summarized in Tables A 4-12 in Annex A (values as found in the interlaboratory test).

9.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limits R as summarized in Tables A 4-12 in Annex A (values as found in the interlaboratory test).

10 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this standard;
- all operating details not specified in this standard, or regarded as optional, together with details of any incidents which may have influenced the test results(s);
- the test result(s) obtained or, if the repeatability has been checked, the final quoted result obtained.

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[3] Directive 2003/115/EC of the European Parliament and of the Council of 22 December 2003 amending Directive 94/35/EC on sweeteners for use in foodstuffs. Official Journal of European Union 2004, L024, 65-71.

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ANNEX A

(informative)

Results of interlaboratory study

The method was validated in a European interlaboratory test with seven participants conducted by the Institute for Reference Materials and Measurements of the European Commission's Directorate General Joint Research Centre in 2007. Method details as applied by the individual laboratories are given in Table A 1. Various beverages and canned fruits differing in fortified concentration amounts of all nine sweeteners were tested in the study (Tables A 2-3); example chromatograms for test samples 1-5 are shown in Figure A 1. Precision data of the individual sweeteners are summarized in Tables A 4-12.

Table A 1. Method conditions applied by individual laboratories

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
SPE characteristics							
- brand name	Chromabond®	Chromabond®	Bakerbond spe®	Chromabond®	Chromabond®	Chromabond®	Chromabond®
- stationary phase	C18ec	C18ec	C18	C18ec	C18ec	C18ec	C18ec
- capacity [mL/mg]	6/1000	6/1000	3/500	6/1000	6/1000	6/1000	6/1000
HPLC apparatus							
- manufacturer	Agilent	Jasco	Shimadzu	Dionex	Jasco	Varian	Dionex
Column characteristics							
- brand name	Purospher® Star	Purospher® Star	Purospher® Star	Nucleodur®	Purospher® Star	Purospher® Star	Purospher® Star
- stationary phase	RP-C18 endcapped	RP-C18 endcapped	RP-C18 endcapped	C-18ec Pyramid	RP-C18 endcapped	RP-C18 endcapped	RP-C18 endcapped
- length [mm]	250	250	250	250	250	250	250
- i.d. [mm]	3	3	3	3	3	3	3
- particle size [µm]	5	5	5	5	5	5	5
HPLC mobile phase							
- mobile phase A composition [v/v/v]	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7	Methanol:Buffer solution:Acetone; 69:24:7
- mobile phase B composition [v/v/v]	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7	Methanol:Buffer solution:Acetone; 11:82:7
- flow rate [mL/min]	0.5	0.5	0.5	0.5	0.6	0.55	0.5
HPLC separation mode							
- gradient program [min - mobile phase A %]	0min - 100% A; 4min - 100% A; 11min - 47% A; 23min - 2% A; 24min -2% A; 26min -100% A	0min - 5% A; 10min - 60% A; 30min - 95% A; 31min - 95 % A; 32min - 5% A; 45min - 5% A	0min - 0% A; 15min - 100% A; 18min - 100 % A; 20min - 0% A; 35min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 21min - 100% A; 23min - 100 % A; 25min - 0% A; 31min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 0% A
HPLC injection mode							
- manual/automatic	automatic	automatic	automatic	automatic	automatic	automatic	automatic
ELSD conditions							
- manufacturer	Sedex 85, Sedere	Varex MKIII, Alltech	ELSD-LT II, Shimadzu	Sedex, Sedere	Sedex 75, Sedere	ELSD 2000ES, Alltech	ELSD 2000ES, Alltech
- drift tube temperature [°C]	40	90	50	43	45	85	85
- nitrogen/air [pressure/flow]	nitrogen 3.2 bar	nitrogen 2.5 L/min	air 3 bar	nitrogen 3.5 bar	air 2.5 bar	nitrogen 2.5 L/min	nitrogen 2.5 L/min
- gain	7	1	9	10	2	1	1

Table A 2. Composition of test samples (beverages) used in the interlaboratory study

Matrix	Beverages				
	Sample 1[(1)]	Sample 2[(2)]	Sample 3[(3)]	Sample 4[(4)]	Sample 5[(5)]
Sweetener	**Fortified concentration in [mg/L]**				
ACS-K	0	42.1	282.5	354.2	421.7
ALI	0	36.5	80.5	102.6	122.2
ASP	0	42.0	485.0	605.0	720.3
CYC	0	36.9	239.0	252.7	300.8
DUL	0	60.7	81.3	101.8	121.1
NEO	0	37.5	80.5	102.2	121.7
NHDC	0	36.7	40.2	50.7	60.4
SAC	0	40.3	65.2	80.9	96.3
SCL	0	38.9	251.8	302.6	360.3

[(1)] Energy drink - blank; [(2)] energy drink fortified at concentration level close to the limit of quantification (LOQs); [(3)] non-carbonated soft drink fortified at a concentration level of ca. 80 % of MUDs; [(4)] carbonated soft drink fortified at a concentration level of ca. 100 % of MUDs; [(5)] carbonated soft drink fortified at a concentration level of ca. 120 % of MUDs

Table A 3. Composition of test samples (canned fruits) used in the interlaboratory study

Matrix	Canned fruits				
	Sample 6[(1)]	Sample 7[(2)]	Sample 8[(3)]	Sample 9[(4)]	Sample 10[(5)]
Sweetener	**Fortified concentration in [mg/kg]**				
ACS-K	0	36.5	265.6	338.8	410.0
ALI	0	34.6	116.1	145.1	175.5
ASP	0	37.3	752.1	967.8	1171.1
CYC	0	32.2	752.6	968.8	1172.3
DUL	0	50.2	114.3	145.7	176.3
NEO	0	36.2	118.3	145.4	175.9
NHDC	0	33.4	37.5	48.9	59.1
SAC	0	38.0	150.0	194.0	234.8
SCL	0	34.6	313.1	388.2	469.7

[(1)] Canned cocktail fruits - blank; [(2)] canned cocktail fruits fortified at concentration level close to the limit of quantification; [(3)] canned pears fortified at a concentration level of ca. 75 % of MUDs; [(4)] canned pears fortified at a concentration level of ca. 100 % of MUDs; [(5)] canned pears fortified at a concentration level of ca. 115 % of MUDs

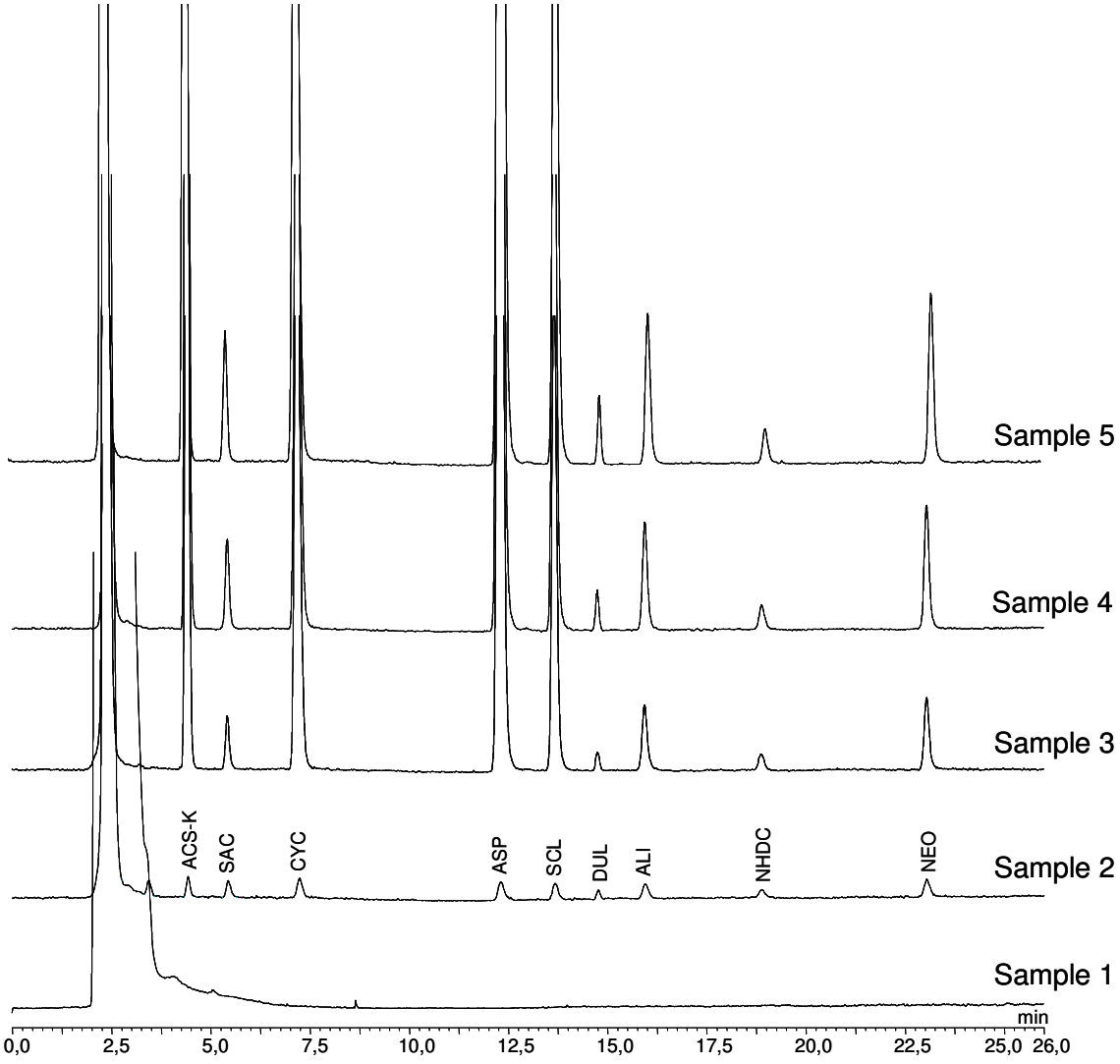

Figure A 1. HPLC-ELSD separations of test samples 1-5 using a fully end-capped reversed phase HPLC column of 250 mm x 3 mm dimensions, particle size 5 µm (Purospher® Star RP-18) from Merck (experimental conditions as described in the method)

Table A 4. Precision data for Acesulfame-K

Sweetener	**Acesulfame-K**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	38.3	266.6	324.1	383.5
True value [mg/L]	42.1	282.5	354.2	421.7
Recovery [%]	90.9	94.4	91.5	90.9
Repeatability standard deviation s_r [mg/L]	2.6	6.0	10.6	9.2
Repeatability relative standard deviation RSD_r [%]	6.9	2.3	3.3	2.4
Repeatability limit r [mg/L]	7.4	16.9	29.7	25.7
Reproducibility standard deviation s_R [mg/L]	4.2	15.6	20.1	19.3
Reproducibility relative standard deviation RSD_R [%]	10.9	5.9	6.2	5.0
Reproducibility limit R [mg/L]	11.6	43.8	56.2	54.0
HorRAT value = RSD_R/predicted RSD_R (1)	1.2	0.9	0.9	0.8
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	1	0
Identity of outlying laboratories			6	
Reason for removal			Co (2)	
Number of accepted laboratories	7	7	6	7
Mean value [mg/kg]	38.4	259.2	323.0	391.3
True value [mg/kg]	36.5	265.6	338.8	410.0
Recovery [%]	105.1	97.6	95.3	95.4
Repeatability standard deviation s_r [mg/kg]	2.7	9.1	4.1	11.4
Repeatability relative standard deviation RSD_r [%]	6.9	3.5	1.3	2.9
Repeatability limit r [mg/kg]	7.4	25.6	11.5	32.0
Reproducibility standard deviation s_R [mg/kg]	5.7	12.7	16.0	17.5
Reproducibility relative standard deviation RSD_R [%]	14.8	4.9	4.9	4.5
Reproducibility limit R [mg/kg]	15.9	35.5	44.8	49.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.6	0.7	0.7	0.7

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; (2) Co = Cochran

Table A 5. Precision data for Alitame

Sweetener	**Alitame**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	31.1	69.1	96.4	114.5
True value [mg/L]	36.5	80.5	102.6	122.2
Recovery [%]	85.3	85.8	93.9	93.7
Repeatability standard deviation s_r [mg/L]	2.2	2.8	2.3	1.5
Repeatability relative standard deviation RSD_r [%]	7.1	4.0	2.3	1.3
Repeatability limit r [mg/L]	6.2	7.7	6.3	4.3
Reproducibility standard deviation s_R [mg/L]	3.0	7.5	2.6	3.9
Reproducibility relative standard deviation RSD_R [%]	9.5	10.9	2.7	3.4
Reproducibility limit R [mg/L]	8.3	21.1	7.2	11.0
HorRAT value = RSD_R/predicted RSD_R (1)	1.0	1.3	0.3	0.4
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	36.0	113.7	142.5	175.2
True value [mg/kg]	34.6	116.1	145.1	175.5
Recovery [%]	104.2	97.9	98.3	99.8
Repeatability standard deviation s_r [mg/kg]	3.5	2.5	3.1	6.4
Repeatability relative standard deviation RSD_r [%]	9.7	2.2	2.2	3.7
Repeatability limit r [mg/kg]	9.7	6.9	8.8	18.0
Reproducibility standard deviation s_R [mg/kg]	3.5	3.8	4.4	7.5
Reproducibility relative standard deviation RSD_R [%]	9.7	3.3	3.1	4.3
Reproducibility limit R [mg/kg]	9.7	10.6	12.3	21.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.0	0.4	0.4	0.6

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table A 6. Precision data for Aspartame

Sweetener	**Aspartame**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	1	0	0	1
Identity of outlying laboratories	3			5
Reason for removal	SG [3]			Co [2]
Number of accepted laboratories	6	7	7	6
Mean value [mg/L]	38.1	485.1	584.8	702.0
True value [mg/L]	42.0	485.0	605.0	720.3
Recovery [%]	90.7	100.0	96.7	97.5
Repeatability standard deviation s_r [mg/L]	1.9	9.5	5.0	5.8
Repeatability relative standard deviation RSD_r [%]	4.9	1.9	0.9	0.8
Repeatability limit r [mg/L]	5.2	26.5	14.1	16.2
Reproducibility standard deviation s_R [mg/L]	6.1	33.3	30.9	23.5
Reproducibility relative standard deviation RSD_R [%]	16.0	6.9	5.3	3.4
Reproducibility limit R [mg/L]	17.1	93.3	86.6	65.9
HorRAT value = RSD_R/predicted RSD_R [1]	1.7	1.1	0.9	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	1	0	2	1
Identity of outlying laboratories	3		4, 6	3
Reason for removal	SG [3]		Co [2]	Co [2]
Number of accepted laboratories	6	7	5	6
Mean value [mg/kg]	37.2	739.8	951.9	1120.2
True value [mg/kg]	37.3	752.1	967.8	1171.1
Recovery [%]	99.9	98.4	98.4	95.6
Repeatability standard deviation s_r [mg/kg]	3.6	16.5	4.5	13.5
Repeatability relative standard deviation RSD_r [%]	9.7	2.2	0.5	1.2
Repeatability limit r [mg/kg]	10.1	46.3	12.5	37.8
Reproducibility standard deviation s_R [mg/kg]	3.6	29.3	27.5	31.7
Reproducibility relative standard deviation RSD_R [%]	9.7	4.0	2.9	2.8
Reproducibility limit R [mg/kg]	10.1	82.0	77.1	88.8
HorRAT value = RSD_R/predicted RSD_R [1]	1.0	0.7	0.5	0.5

[1] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [2] Co = Cochran; [3] SG = Single Grubbs

Table A 7. Precision data for Cyclamate

Sweetener	**Cyclamate**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	28.3	248.9	256.8	307.2
True value [mg/L]	36.9	239.0	252.7	300.8
Recovery [%]	76.8	104.1	101.6	102.1
Repeatability standard deviation s_r [mg/L]	1.2	6.6	3.6	5.9
Repeatability relative standard deviation RSD_r [%]	4.4	2.6	1.4	1.9
Repeatability limit r [mg/L]	3.5	18.4	10.2	16.5
Reproducibility standard deviation s_R [mg/L]	5.8	15.4	14.0	15.5
Reproducibility relative standard deviation RSD_R [%]	20.6	6.2	5.5	5.0
Reproducibility limit R [mg/L]	16.3	43.1	39.2	43.4
HorRAT value = RSD_R/predicted RSD_R (1)	2.1	0.9	0.8	0.7
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	1
Identity of outlying laboratories		3		5
Reason for removal		Co (2)		Co (2)
Number of accepted laboratories	7	6	7	6
Mean value [mg/kg]	27.5	749.7	924.7	1100.6
True value [mg/kg]	32.2	752.6	968.8	1172.3
Recovery [%]	85.2	99.6	95.5	93.9
Repeatability standard deviation s_r [mg/kg]	4.4	7.0	14.5	12.7
Repeatability relative standard deviation RSD_r [%]	16.1	0.9	1.6	1.2
Repeatability limit r [mg/kg]	12.4	19.6	40.5	35.6
Reproducibility standard deviation s_R [mg/kg]	4.9	30.9	44.4	37.2
Reproducibility relative standard deviation RSD_R [%]	17.9	4.1	4.8	3.4
Reproducibility limit R [mg/kg]	13.7	86.5	124.2	104.3
HorRAT value = RSD_R/predicted RSD_R (1)	1.8	0.7	0.8	0.6

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; (2) Co = Cochran

Table A 8 Precision data for Dulcin

Sweetener	**Dulcin**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	55.0	79.6	95.7	115.1
True value [mg/L]	60.7	81.3	101.8	121.1
Recovery [%]	90.6	98.0	94.0	95.0
Repeatability standard deviation s_r [mg/L]	1.4	2.9	1.0	1.5
Repeatability relative standard deviation RSD_r [%]	2.5	3.7	1.0	1.3
Repeatability limit r [mg/L]	3.8	8.2	2.8	4.3
Reproducibility standard deviation s_R [mg/L]	3.3	3.9	5.2	5.2
Reproducibility relative standard deviation RSD_R [%]	6.1	4.9	5.5	4.6
Reproducibility limit R [mg/L]	9.4	10.9	14.7	14.7
HorRAT value = RSD_R/predicted RSD_R [(1)]	0.7	0.6	0.7	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	1	0	0	0
Identity of outlying laboratories	6			
Reason for removal	NC [(2)]			
Number of accepted laboratories	6	7	7	7
Mean value [mg/kg]	49.8	111.0	141.7	172.6
True value [mg/kg]	50.2	114.3	145.7	176.3
Recovery [%]	99.3	97.0	97.3	97.9
Repeatability standard deviation s_r [mg/kg]	3.7	3.0	3.6	3.1
Repeatability relative standard deviation RSD_r [%]	7.4	2.7	2.5	1.8
Repeatability limit r [mg/kg]	10.3	8.4	10.1	8.6
Reproducibility standard deviation s_R [mg/kg]	4.3	4.8	4.7	5.4
Reproducibility relative standard deviation RSD_R [%]	8.6	4.3	3.3	3.1
Reproducibility limit R [mg/kg]	12.0	13.4	13.1	15.2
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.0	0.5	0.4	0.4

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] NC = Non compliant data

Table A 9. Precision data for Neotame

Sweetener	**Neotame**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	37.6	77.9	97.2	115.3
True value [mg/L]	37.5	80.5	102.2	121.7
Recovery [%]	100.1	96.8	95.1	94.7
Repeatability standard deviation s_r [mg/L]	0.9	1.9	2.4	2.8
Repeatability relative standard deviation RSD_r [%]	2.3	2.4	2.4	2.4
Repeatability limit r [mg/L]	2.4	5.2	6.7	7.7
Reproducibility standard deviation s_R [mg/L]	2.4	4.6	4.8	5.2
Reproducibility relative standard deviation RSD_R [%]	6.4	5.9	5.0	4.5
Reproducibility limit R [mg/L]	6.8	12.9	13.5	14.4
HorRAT value = RSD_R/predicted RSD_R (1)	0.7	0.7	0.6	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	37.3	116.2	140.6	173.7
True value [mg/kg]	36.2	118.3	145.4	175.9
Recovery [%]	103.0	98.2	96.7	98.7
Repeatability standard deviation s_r [mg/kg]	1.3	3.6	2.2	4.8
Repeatability relative standard deviation RSD_r [%]	3.5	3.1	1.6	2.8
Repeatability limit r [mg/kg]	3.6	10.1	6.2	13.5
Reproducibility standard deviation s_R [mg/kg]	2.2	6.3	7.5	7.7
Reproducibility relative standard deviation RSD_R [%]	5.9	5.4	5.3	4.5
Reproducibility limit R [mg/kg]	6.2	17.6	21.1	21.7
HorRAT value = RSD_R/predicted RSD_R (1)	0.6	0.7	0.7	0.6

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table A 10 Precision data for Neohesperidine dihydrochalcone

Sweetener	**Neohesperidine dihydrochalcone**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	31.4	42.8	51.0	59.3
True value [mg/L]	36.7	40.2	50.7	60.4
Recovery [%]	85.5	106.4	100.5	98.2
Repeatability standard deviation s_r [mg/L]	3.3	1.7	1.8	2.6
Repeatability relative standard deviation RSD_r [%]	10.6	3.9	3.5	4.4
Repeatability limit r [mg/L]	9.3	4.7	4.9	7.3
Reproducibility standard deviation s_R [mg/L]	9.0	6.7	4.4	5.2
Reproducibility relative standard deviation RSD_R [%]	28.5	15.6	8.7	8.8
Reproducibility limit R [mg/L]	25.1	18.7	12.4	14.5
HorRAT value = RSD_R/predicted RSD_R (1)	3.0	1.7	1.0	1.0
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	0
Identity of outlying laboratories		5		
Reason for removal		Co (2)		
Number of accepted laboratories	7	6	7	7
Mean value [mg/kg]	35.3	40.5	49.8	59.3
True value [mg/kg]	33.4	37.5	48.9	59.1
Recovery [%]	105.6	108.0	102.0	100.4
Repeatability standard deviation s_r [mg/kg]	2.2	1.0	2.0	2.3
Repeatability relative standard deviation RSD_r [%]	6.1	2.5	4.0	3.9
Repeatability limit r [mg/kg]	6.1	2.8	5.6	6.5
Reproducibility standard deviation s_R [mg/kg]	4.4	4.6	3.3	5.5
Reproducibility relative standard deviation RSD_R [%]	12.4	11.5	6.6	9.2
Reproducibility limit R [mg/kg]	12.2	13.0	9.2	15.3
HorRAT value = RSD_R/predicted RSD_R (1)	1.3	1.3	0.7	1.1

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; (2) Co = Cochran

Table A 11. Precision data for Saccharin

Sweetener	**Saccharin**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	1
Identity of outlying laboratories		6		6
Reason for removal		Co [(2)]		Co [(2)]
Number of accepted laboratories	7	6	7	6
Mean value [mg/L]	36.2	60.1	74.1	87.6
True value [mg/L]	40.3	65.2	80.9	96.3
Recovery [%]	89.8	92.1	91.5	91.0
Repeatability standard deviation s_r [mg/L]	1.4	1.7	3.0	1.0
Repeatability relative standard deviation RSD_r [%]	3.8	2.8	4.0	1.1
Repeatability limit r [mg/L]	3.9	4.7	8.3	2.7
Reproducibility standard deviation s_R [mg/L]	4.0	2.8	4.9	5.2
Reproducibility relative standard deviation RSD_R [%]	11.1	4.6	6.6	5.9
Reproducibility limit R [mg/L]	11.3	7.7	13.6	14.5
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.2	0.5	0.8	0.7
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	44.3	151.9	193.4	235.3
True value [mg/kg]	38.0	150.0	194.0	234.8
Recovery [%]	116.7	101.3	99.7	100.2
Repeatability standard deviation s_r [mg/kg]	2.4	4.0	4.3	6.7
Repeatability relative standard deviation RSD_r [%]	5.5	2.7	2.2	2.9
Repeatability limit r [mg/kg]	6.8	11.3	12.0	18.8
Reproducibility standard deviation s_R [mg/kg]	8.4	10.6	13.5	15.0
Reproducibility relative standard deviation RSD_R [%]	19.0	7.0	7.0	6.4
Reproducibility limit R [mg/kg]	23.6	29.6	37.7	42.0
HorRAT value = RSD_R/predicted RSD_R [(1)]	2.1	0.9	1.0	0.9

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] Co = Cochran

Table A 12. Precision data for Sucralose

Sweetener	**Sucralose**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	36.8	245.1	282.9	346.8
True value [mg/L]	38.9	251.8	302.6	360.3
Recovery [%]	94.7	97.3	93.5	96.3
Repeatability standard deviation s_r [mg/L]	1.4	3.8	2.7	8.2
Repeatability relative standard deviation RSD_r [%]	3.7	1.5	0.9	2.4
Repeatability limit r [mg/L]	3.8	10.6	7.4	22.9
Reproducibility standard deviation s_R [mg/L]	5.2	10.1	16.2	13.3
Reproducibility relative standard deviation RSD_R [%]	14.2	4.1	5.7	3.8
Reproducibility limit R [mg/L]	14.7	28.2	45.3	37.4
HorRAT value = RSD_R/predicted RSD_R (1)	1.5	0.6	0.8	0.6
Sample Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	35.3	306.1	380.2	462.4
True value [mg/kg]	34.6	313.1	388.2	469.7
Recovery [%]	102.1	97.7	98.0	98.4
Repeatability standard deviation s_r [mg/kg]	2.2	7.4	8.5	9.7
Repeatability relative standard deviation RSD_r [%]	6.3	2.4	2.2	2.1
Repeatability limit r [mg/kg]	6.3	20.6	23.8	27.1
Reproducibility standard deviation s_R [mg/kg]	3.8	8.7	10.4	9.7
Reproducibility relative standard deviation RSD_R [%]	10.9	2.8	2.7	2.1
Reproducibility limit R [mg/kg]	10.8	24.4	29.1	27.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.2	0.4	0.4	0.3

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

ANNEX B

(informative)

Calibration graphs of individual sweeteners

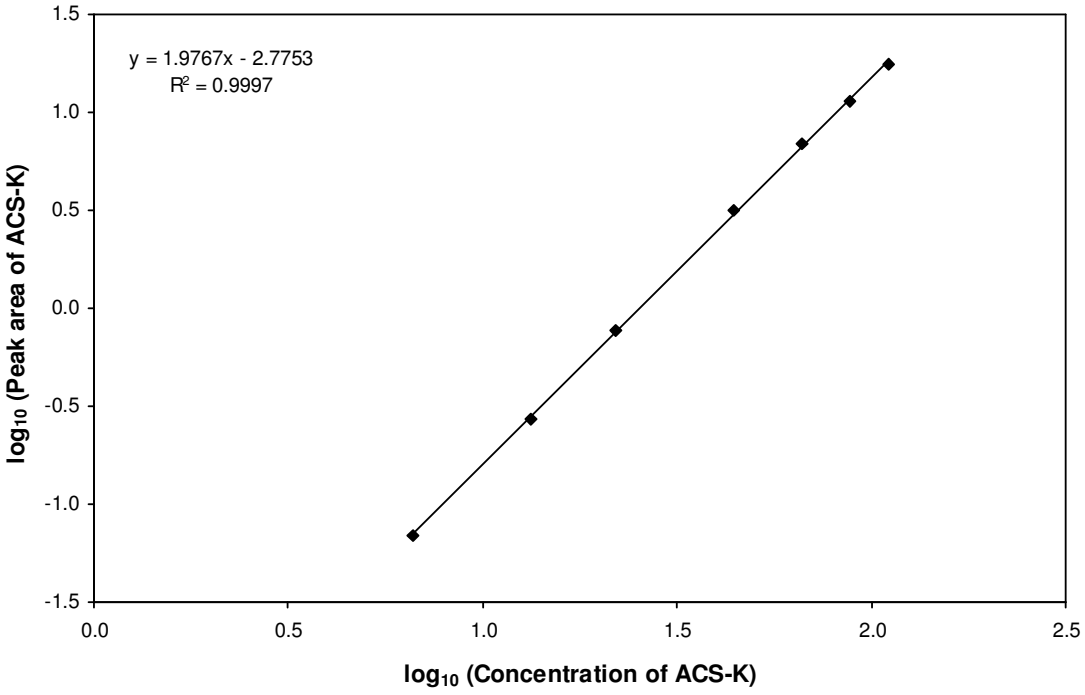

Figure B 1. Seven point calibration graph of ACS-K as obtained by one of the laboratories participating in the collaborative study

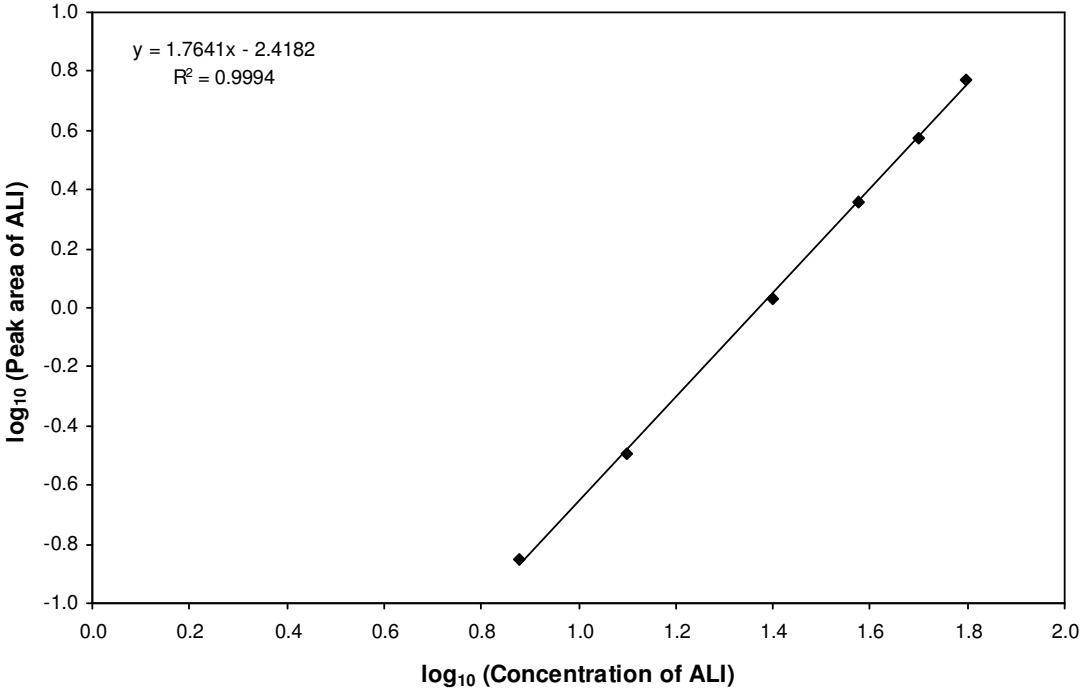

Figure B 2. Six point calibration graph of ALI as obtained by one of the laboratories participating in the collaborative study

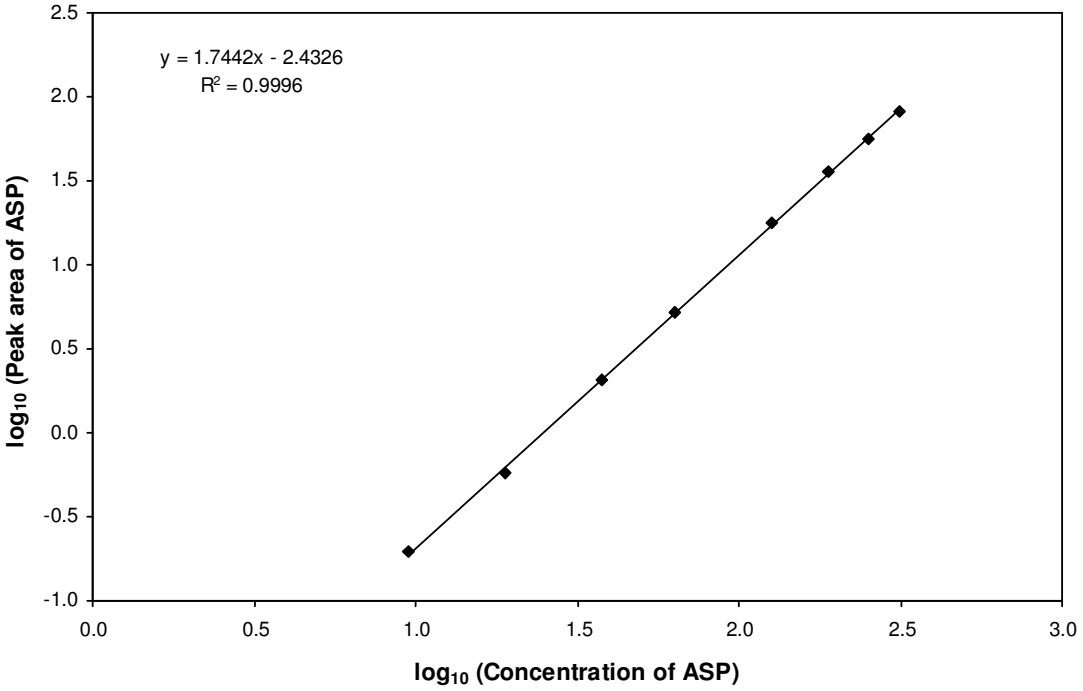

Figure B 3. Eight point calibration graph of ASP as obtained by one of the laboratories participating in the collaborative study

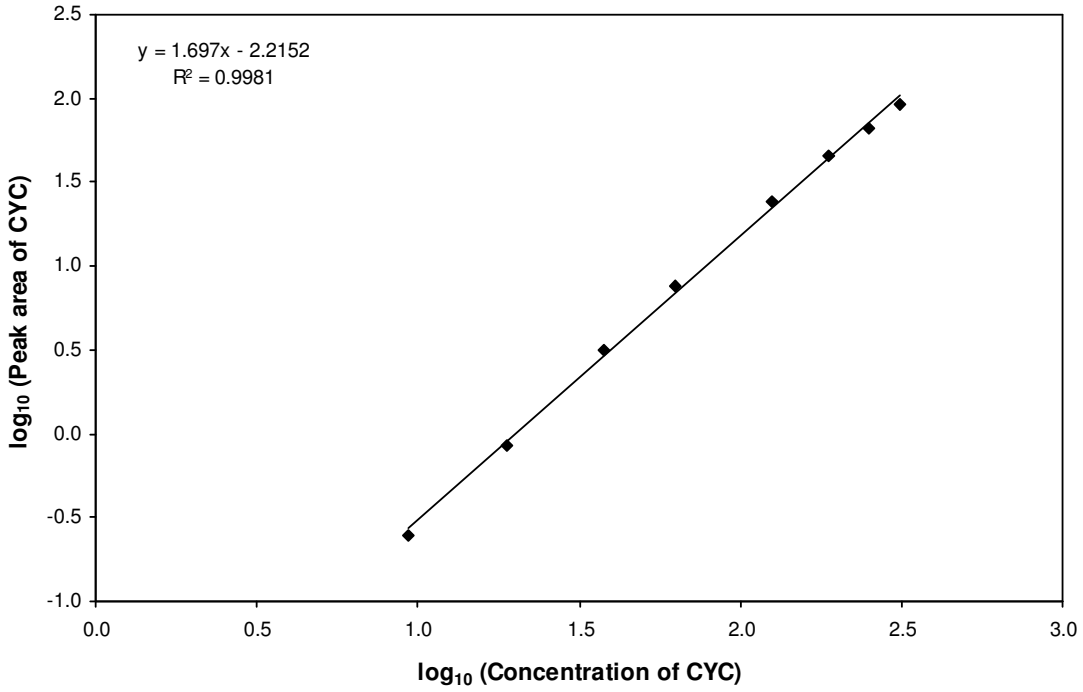

Figure B 4. Eight point calibration graph of CYC as obtained by one of the laboratories participating in the collaborative study

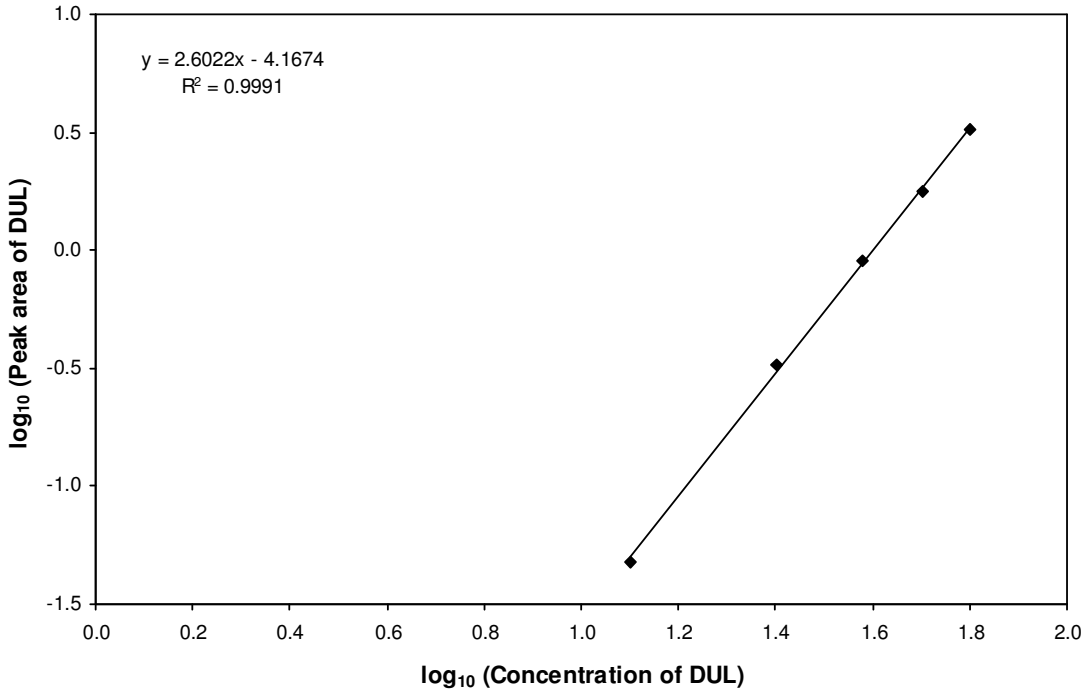

Figure B 5. Five point calibration graph of DUL as obtained by one of the laboratories participating in the collaborative study

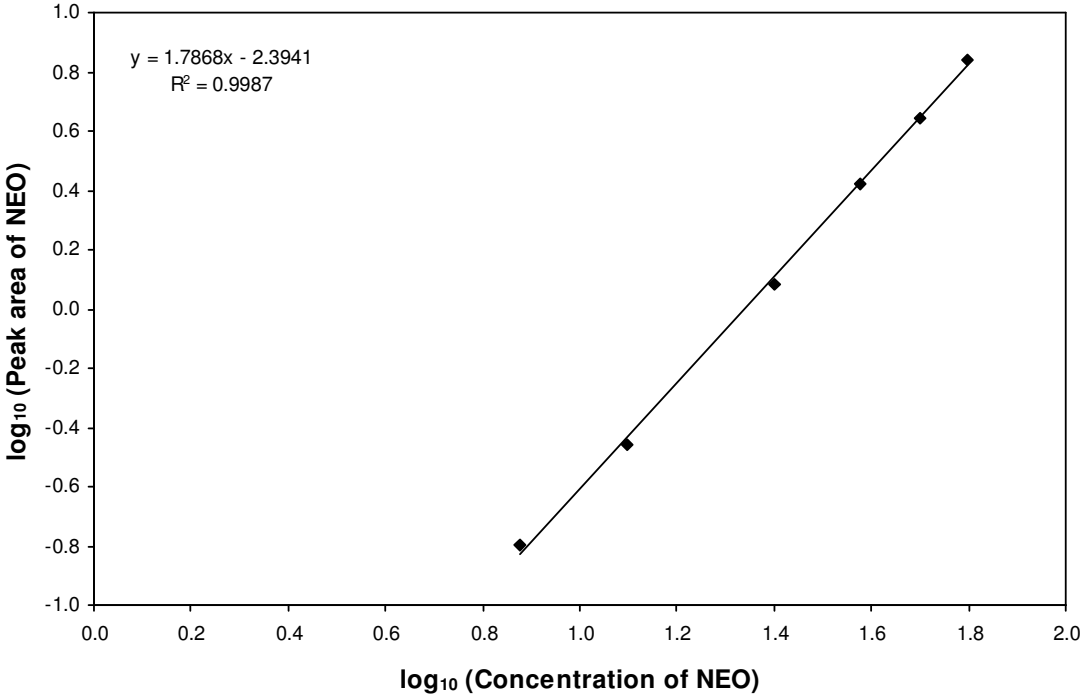

Figure B 6. Six point calibration graph of NEO as obtained by one of the laboratories participating in the collaborative study

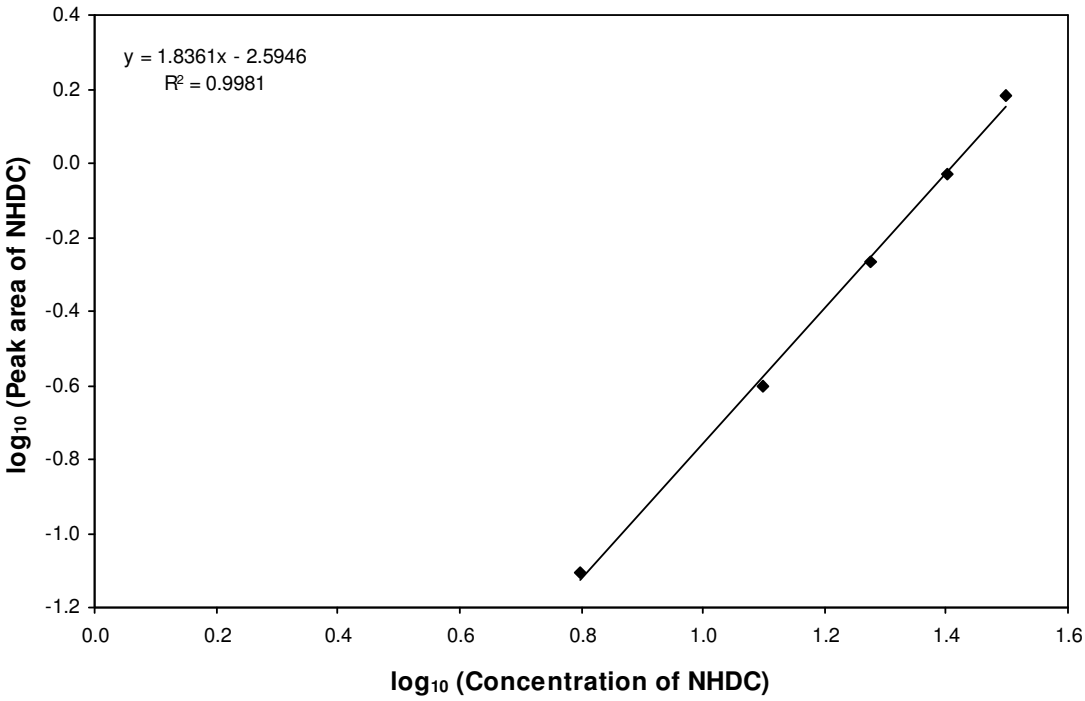

Figure B 7. Five point calibration graph of NHDC as obtained by one of the laboratories participating in the collaborative study

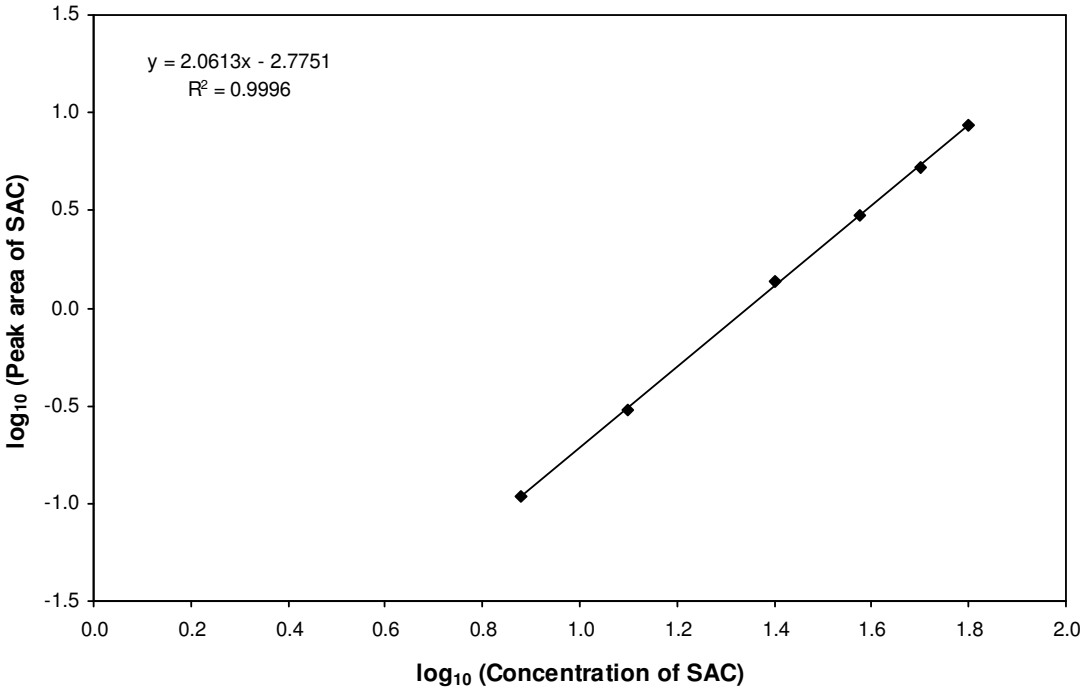

Figure B 8. Six point calibration graph of SAC as obtained by one of the laboratories participating in the collaborative study

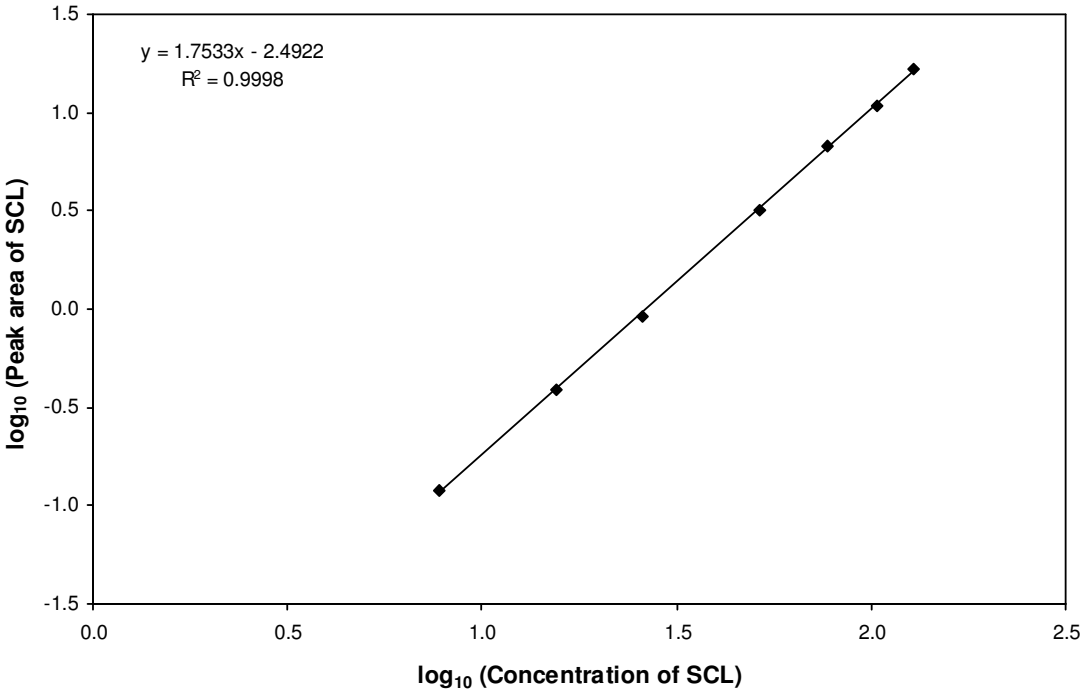

Figure B 9. Seven point calibration graph of SCL as obtained by one of the laboratories participating in the collaborative study

ANNEX C

(informative)

Typical chromatogram for calibration standard

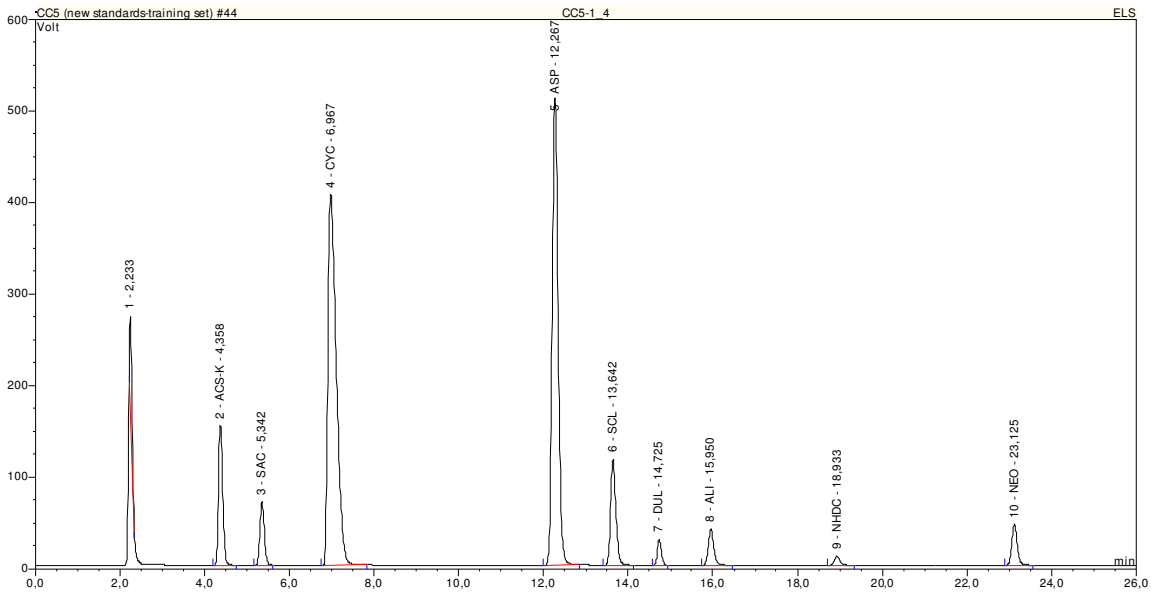

Figure C 1. Chromatographic separation of all nine sweeteners obtained by analysis of calibration solution 1 (3.19)

European Commission

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